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Respectfully submitted,

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Table 2. Comparison of bhpW and peptides based on the CD4 C'-C'' loop

Peptide	Ceff (mM)	[θ]215 deg cm ² dmol ⁻¹	No. of ³ J _{HN} H α > 8 Hz	δ (Cys _N H α) (ppm)	δ (Cys _C H α) (ppm)
Ac-CTWEGNKLTC-NH ₂ <i>bhpW</i> (SEQ ID NO: 2)	210 \pm 4	- 19,800	7	5.20	5.00
SCTWEGNKLTC-NH ₂ (SEQ ID NO: 3)	273 \pm 2	- 17,400	n. d.	n. d.	n. d.
Ac-CGNQGSFLTC-NH ₂ <i>cd1</i> (SEQ ID NO: 4)	n. d.	n. d.	0	4.66	4.66
Ac-CTWQGSFLTC-NH ₂ <i>cd2</i> (SEQ ID NO: 5)	n. d.	- 15,800	6	5.08	4.93
SCGNQGSFLTCK-NH ₂ <i>cd1a</i> (SEQ ID NO: 6)	45 \pm 4	- 1,500	0	4.80	4.72
SCTNQGSFLTCK-NH ₂ (SEQ ID NO: 7)	n. d.	- 5,000	2	4.96	4.79
SCGWQGSFLTCK-NH ₂ (SEQ ID NO: 8)	48 \pm 0	- 6,100	3	5.00	4.88
SCTWQGSFLTCK-NH ₂ <i>cd2a</i> (SEQ ID NO: 9)	120 \pm 0	- 14,000	6	5.36	5.14

Terminal serine and lysine residues were added to improve the solubility of some variants of the CD4 peptide, which are otherwise uncharged. A similar modification was made to bhpW as a control. Non-turn residues that differ between bhpW and the CD4 loop are underlined. Coelution of reduced and oxidized peptides prevented measurement of C_{eff} for the T2, N3 variant of the CD4 peptide. Circular dichroism spectra were acquired at 10 °C with an Aviv Instruments, Inc. Model 202 spectrophotometer; peptide concentrations were 20 μ M in 20 mM potassium phosphate, pH 7.0.

[n. d.: not determined; Cys_N H^α: H^α chemical shift for the more N-terminal cysteine (Cys1 or Cys2); Cys_C H^α: H^α chemical shift for the more C-terminal cysteine (Cys10 or Cys11)]

Two other turn sequences evaluated were VWQL from the F-G loop of domain 2 of human Fc-epsilon-RI, and GPLT from the EPO agonist peptide EMP1. All three turns were evaluated in the trp peptide scaffold and in cyclized peptides whose sequence matched more closely the native parent hairpin loops:

	SCGNQGSFLTCK-NH ₂	CD4 peptides	a
	(SEQ ID NO: 10)		
5	SCTWQGSFLTCK-NH ₂	(SEQ ID NO: 11)	b
	Ac-CTKVWQLWTC-NH ₂	Fc-epsilon-RI peptides	c
	(SEQ ID NO: 12)		
	SCTWVWQLLTCK-NH ₂	(SEQ ID NO: 13)	d
	SCHFGPLTWVCK-NH ₂	EMP1 peptides	e
	(SEQ ID NO: 14)		
	SCTWGPLTLTCK-NH ₂	(SEQ ID NO: 15)	f

10 Circular dichroism spectra show that in each case, the designed trp hairpin scaffold yields a more structured peptide (Fig. 5a-c). NMR data are consistent with increased hairpin structure in the peptides, demonstrating that the scaffold can bias a variety of "difficult" turns toward structured states.

Other common turns that can be presented on the hairpin scaffold include gamma-turns (3 amino acids), bulged turns (5 or 6 amino acids), and longer hairpins (8 amino acids). Other turn lengths are known and are also
15 be compatible with the scaffold.

The results in Example 1 and 2 demonstrate that optimization of a single strand position in a small disulfide-constrained hairpin is sufficient to convert a very poorly structured molecule to one that is highly structured ($-\Delta\Delta G > 0.8$ kcal / mol). The stem portion of the structured hairpin, -CTW---LTC-, does not require an optimized turn sequence; thus, it is a suitable scaffold for display of β -turn libraries and for studying
20 particular turns that might not otherwise be highly populated. Importantly, only natural amino acids are required, so turn libraries may be displayed on phage.

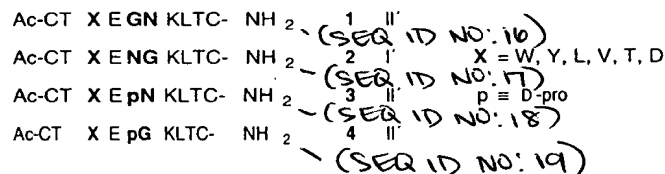
It is interesting to compare the substitution energies we report here with previous studies on β -sheet systems. Although the magnitude of the energy differences is similar, the rank order we obtain does not correlate with experimental β -propensity scales or with observed residue pair frequencies in known β -sheets (Hutchinson
25 *et al.* (1998) *Protein Sci.* 7:2287-2300, Wouters, M. A. & Curmi, P. M. G. (1995) *Proteins* 22:119-131). In particular, tryptophan is unexceptional in such scales. These differences stress that average trends in typical protein domains may not apply directly to small peptides in which most residues are highly solvent exposed, complicating the use of such information in *de novo* design. Furthermore, $\Delta\Delta G$ rank order does not correlate well with increasing non-polar surface area of the side chains, although the preferred residues are hydrophobic.

30 Finally, the hairpin stem is very small, yet the combination of disulfide and cross-strand tertiary contact imparts a structural bias exceeding that of a disulfide alone, e. g. CX₄C. Although it is known that some particular sequences (e. g., VVVV) (Milburn *et al.* (1988) *Int. J. Peptide Protein Res.* 31:311-321.) cannot adopt turn conformations compatible with our hairpin, it is also true that very few of the turn sequences observed in

proteins have been shown to adopt well defined turn conformations in isolated peptides. We have demonstrated a simple strategy to increase this number. We envision that hairpin libraries with randomized turn sequences (e. g., XCTWX₄LTCX) might yield structured ligands whose binding determinants could be transferred readily to small synthetic turn mimetics or even used directly to identify small-molecule leads for high-throughput affinity optimization (Rohrer *et al.* (1998) *Science* **282**:737-740).

Example 3: Quantification of the Relative Contributions from Turns and Cross-Strand Interactions

In Example 1 above, substitutions were introduced into position 3 (X) of the model peptide **bhp** (peptide 1). This guest site is quite close in space to the type II' turn (gly-asn, Fig. 1). To further investigate whether hairpins with different turn sequences and geometries would have different residue preferences at the NHB guest site, the central gly-asn sequence in model peptide 1 is replaced with the type I' turn asn-gly (peptide 2) and the type II' turns D-pro-asn and D-pro-gly (peptides 3 and 4). Substitutions at position 3 (X) were chosen to span the range of hairpin stabilities we observed in the gly-asn series. C_{eff} was measured as previously described in Example 1. The values we obtain for the different turns are compared in Figure 6.



In all cases, tryptophan at position 3 yields the largest C_{eff} value for a given turn, demonstrating that its stabilizing influence is general. The large changes in C_{eff} for the different cross-strand interactions (horizontal axis) and turn sequences (vertical axis) show that both can contribute significantly to stability in these cyclic hairpin peptides. Finally, there are striking linear correlations between data sets, indicating that substitutions at strand position 3 and the turn replacements make independent contributions to stability of the cyclic hairpin. These data suggest that the hairpin fold may be quite modular, which would significantly simplify hairpin design.

Relative turn energies can be calculated by comparing C_{eff} for the appropriate pairs of peptides. However, the correlation in Figure 6 allow the calculation of relative turn energies from the slopes, which should be less sensitive to experimental error. These values are listed in Table 3. Compared to asn-gly (type I'), gly-asn (type II') is less stabilizing, while the D-pro-containing turns (also type II') enhance hairpin stability. In the one case where a comparison may be made, asn-gly vs. D-pro-gly, the $\Delta\Delta G$ value obtained here agrees reasonably well with that obtained by NMR. This suggests that the reference states assigned by Syud *et al.* (1999) *J Am Chem Soc* **121**:11577 and their assumption of two-state folding are appropriate for their model system; however, defining such reference states is not always feasible.



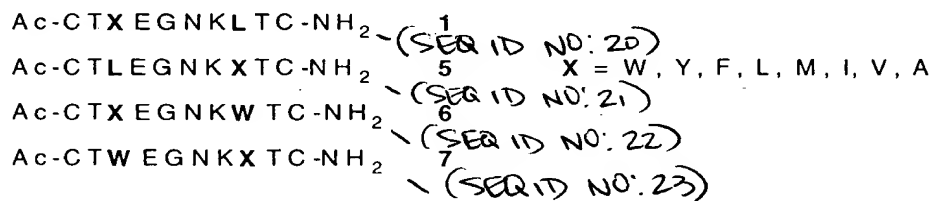
In order to assess how the turn types affect the hairpin structure, the tryptophan analogs of **2** and **3** were characterized by NMR spectroscopy, and structures were calculated as described in Example 1 for peptide **1**. The comparison of minimized mean structures in Figure 7 reveals that the backbone and side chain conformations are very similar for the non-turn residues (RMSD ~ 0.3 Å) regardless of the type of turn present. Thus, consistent with the linear correlations in C_{eff} (Fig. 6), these three turns do not exert any structural influence on the adjacent strands.

The importance of the turn sequence and good cross-strand pairing to hairpin structure has been addressed in many model studies. However, there is little quantitative data or systematic evaluation of residue substitutions. Our data show that, for these simple cyclic peptides, substitutions in a strand site and in the turn conform to simple linear free-energy relationships and have independent and additive effects on hairpin stability. This is unexpected, given their proximity in the structure (Figs. 3 and 7) and the reported sensitivity of calculated turn energies to features of the protein anchorage (Freidinger, R. M. (1999) *Curr. Opin. Chem. Biol.* 3:395-406). Nonetheless, it would appear that coupling between these turns and the strands is negligible compared to the large influence each exerts alone. This suggests that β -hairpin stability may be understood by separate analysis of these components.

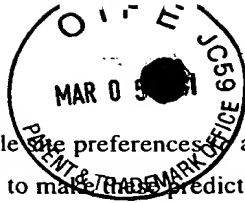
Example 4: Quantification of Energetic Contributions from Cross-Strand Residues

The results of above Examples revealed tryptophan to be quite stabilizing in the non hydrogen-bonded (NHB) strand site X of peptide **1**, when paired with a cross-strand leucine. The tryptophan peptide (bhpW) was highly structured in water, adopting the intended hairpin conformation (Fig. 3). Here we investigate the relationship between the NHB cross-strand residues. Remarkably, we find that residue preferences for the two structurally inequivalent sites are the same, and that specific pair interactions produce only minor deviations from the single site contributions. Accordingly, a tryptophan-tryptophan cross-strand pair appears to be optimal for hairpin stability.

Our observation of a stabilizing contribution from tryptophan prompted us to question how general the effect might be. The tryptophan in peptide bhpW (Fig. 3) is spatially near both the cross-strand leucine and the side chains of residues in the type II' turn. Therefore, it seemed possible that the effect of tryptophan might depend on stabilizing contacts with these other residues. In order to address this question, we reversed the hydrophobic pairs (peptide **5**), varying the amino acid at position 8 (nearest the disulfide, Fig. 3) with leucine fixed at position 3. Effective concentrations (C_{eff}) of the cysteine thiols were determined as in our previous studies.



We find that tryptophan at position 8 is the most stabilizing of those residues tested (Fig. 8A). Significantly, the C_{eff} values are quite close for the trp-leu and leu-trp pairs, indicating that the two arrangements



the combined single site preferences (α and ρ) are most important in predicting hairpin stability. Significantly, it should be possible to make these predictions from a limited basis set of experimental data.

Example 5: Construction of phage-displayed libraries based on the trp peptide scaffold

Libraries of random peptides fused to the gene 8 protein of the filamentous bacteriophage M13 were produced by Kunkel mutagenesis of plasmid pS1302b, a derivative of pS349 (U.S. patent application Nos. 60/103,514 and 60/134,870, incorporated herein by reference). Plasmid pS1302b includes the tac promoter and malE leader sequence of pS349. The hGH sequence and Gly/Ser-rich linker sequence of pS349 were replaced by the sequence:

5'-TAA-TAA-TAA-ATG-GCT-GAT-CCG-AAC-CGT-TTC-CGC-GGT-AAA-GAT-CTG-GGT-GGC-
GGT-ACT-CCA-AAC-GAC-CCG-CCA-ACC-ACT-CCA-CCA-ACT-GAT-AGC-CCA-GGC-GGT-3' (SEQ ID
NO:)

24

The inserted sequence encodes three stop codons, the GD epitope tag, and a linker selected for high-level display of hGH. The plasmid also includes the lac repressor (*lacIq*) and the ampicillin resistance gene from pS349. The oligonucleotide used to construct the library was:

5'-TCC-GCC-TCG-GCT-TAT-GCA-NNS-TGC-ACT-TGG-NNS-NNS-NNS-CTG-ACT-TGT-
NNS-ATG-GCT-GAT-CCG-AAC-CGT-3' (SEQ ID NO:)

25

The form of the random peptides was therefore XCTWX₄LTCX. A library of 10⁹ to 10¹⁰ individual transformants was prepared by previously described methods (U.S. patent application Nos. 60/103,514 and 60/134,870). Approximately one-third of individual clones encoded a functional peptide sequence. The remainder were starting template, contained stop codons, or contained single nucleotide deletions. The library size is thus adequate to include several copies of each possible random sequence.

Example 6: Selection of binding peptides from the structurally-biased library

Nunc MaxiSorp plates were coated overnight with 2 µg/mL rhuFc-epsilon-RI-IgG fusion in PBS. Plates were then blocked for one hour at room temperature with 0.5% BSA (Sigma A-7638) in PBS. Negative wells were prepared by coating only with 0.5% BSA. Phage (10¹¹ i.u. per well) were added to ten each positive and negative wells and incubated with shaking for 20 h at room temperature. After extensive washing to remove nonspecifically-bound phage, binders were eluted by treatment with 0.2 M glycine, pH 2 for five minutes. The eluted phage were then neutralized by addition of TRIS base and used to infect a culture of E. coli (XL1-blue, Stratagene). Several cycles of binding, elution, and amplification (3-5 total) were conducted under similar conditions. 192 individual clones were screened for binding to the target receptor by incubation of phage supernatant with plates prepared as described for phage sorting. After washing, wells were treated with alpha-M13-HRP conjugate (Pharmacia Biotech 27-9421-01), and bound antibody was detected with OPD substrate (Sigma P-9187). Plate absorbance (A₄₉₂-A₄₀₅) was compared between positive and negative plates to identify those clones positive for receptor binding. Twelve such clones were identified.